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Mycotoxin T2, monoclonal anti-T2, EIA, purification, affinity chromatography, immunization, anti-idiotypic antibodies, monoclonal anti-idiotypic antibody, leucopenia, treatment, vaccine, prophylaxis.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An EIA assay system to detect and titer antibody to Mycotoxin T2 was standardized. A new rapid simple method of making T2 hemisuccinate (T2-HS) was developed. The merit of the new method over the existing one accounts for its simple, less time-consuming procedure, which can be carried out at room temperature, with as high a yield as 66%. T2-HS so developed was conjugated to derivatised sepharose-4B beads to make a column to affinity purify the antibodies to Mycotoxin T2. Affinity purified antibody to T2 was used for immunizing the mice for the development of anti-idiotypic antibodies. Three different

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immunization schedules were employed, such as, anti-T2 antibody conjugated to KLH, anti-T2 antibody conjugated to goat IgD, and anti-T2 antibody conjugated to monoclonal B6 anti-BALB/C IgD. Anti-idiotypic antibody was detected by EIA (competitive inhibition reaction by T2). Mice producing a titer of 1:800 to 1:20,000 were detected. These immunization schedules are now being extended to additional mice and rats. Production of hybridoma cell lines, by fusing the immune spleenocytes with NS-1 are initiated.

Induction of leucopenia by T2 toxin in mice was examined. Optimum sublethal concentrations of T2 toxin and route of administration were standardized. Time course of white blood cell count after administration of toxin, was established. Effect of toxin on the other lymphatic organs was also examined.

Annual Report

DEVELOPMENT OF VACCINES TO THE MYCOTOXIN T2

ONR Contract No. N0014-83-C-0441

Introduction

This report presents the work carried out during the 12 months period from May 1983 on ONR Coutract No. N0014-83-C-0441; Development of vaccines to mycotoxin T2. T2 is a trichothecene mycotoxin which is both acutely and chronically toxic. Its adverse effects range from skin necrosis to severe leukopenia and death, and the compound is also a potent immunotoxin. Currently there are no known prophylactic or therapeutic treatments for T2 poisoning other than removal of the compound or prevention of exposure. This project is designed to explore the possibility that anti-idiotypic antibodies (anti-id-ab) to anti-T2 antibodies can be used in an immunization protocol to prevent the adverse effects of T2.

Development of Monoclonal Antibodies tc T2:

Being a small, non-immunogenic molecule, T2 has to be presented as a hapten-carrier conjugate for immunization. Initially we proposed to couple several protein and carbohydrate carriers to T2 toxin through its various chemical moieties including the epoxide group. However, the task of haptenization and immunization of T2, was not carried out, as this work had already been carried out at USUHS by Drs. Hunter and Finkelman, and we were advised by our project officer to collaborate with them. Initially we could obtain a small sample of polyclonal antibody containing serum from them, as monoclonals were not yet produced.

Development of an Assay System:

Our proposed approach was to develop a radioimmunosassay system using [^{3}H]-T2. Experiments using [^{3}H]-T2 (a gift by Dr. Wannamaker, USAMARID) and polyclonal antibody (povided by Dr. Hunter) did not demonstare any activity. Modifications of the assay system such as incubation time, temperature, use of

anti-Fab alone as a second antibody or Cowan's Staph A as the precipitating agent or using both anti-Fab and the Cowan's Staph A, did not precipitate any radiolabeled material.

As the polyclonal antibody supplied by r. Hunter was detected and titered by an enzyme-linked immunoassay (EIA), we decided to adopt and standardize the optimum conditions required for the EIA. After initial screening with several commercially available brands of EIA plates, second antibody reagents, optimum conditions necessary for EIA were determined. These include coating the plates with 25 µg/ml of T2-BSA, blocking the unbound sites with 100 µg/ml of poly L glutamine, and using an affinity purified anti-Fab (a generous gift from Dr. McClintock, NIDR, NIH). Subsequent characterizations of monoclonal and polyclonal sera were carried out using the above conditions.

Purification of Polyclonal and Monoclonal Antibody:

Simultaneously working with the standardization of the assay system to detect anti-T2 antibody in the antiserum, the possibility of making an affinity column to purify the polyclonal antiserum to T2, was also explored. At this point affinity purified anti-T2 antibody was intended to use for the development of anti-idiotypic antisera.

In our earlier experiments to make hapten-carrier conjugates, we had focussed on the development of a T2 hemisuccinate (T2-HS), which can then be coupled through a carbodiimide reaction to amino groups on proteins and other molecules. Experiments were performed according to Chu et al (1977), where in T2 toxin spiked with a small amount of [3H]-T2 was dissolved in dimethyl-formamide and reacted with succinic anhydride in the presence of pyridine. The reaction was carried out in dark at 100°C in a paraffin oil bath with constant stirring for 4 hours. The chloroform extract obtained after decolorising and washing the reaction mixture, yielded 2% of T2 toxin in T2-HS form.

In order to overcome some of the draw backs encountered in this method such as high temperature, duration of reaction procedure, discoloration of the product and others, we developed a new rapid and simple method of T2-HS formation. This reaction can be carried out at room temperature, in a chloroform phase, without heating. Under optimal conditions complete

conversion of T2 to T2-HS can be achieved within 2 hours. The resultant product which is in chloroform phase, on drying yields a coloreless powder with a recovery rate of 66% as determined by spiking with [^{3}H] T2 toxin.

The T2 HS, prepared by the new method was conjugated to derivatised sepharose 4B beads (Sigma), at room temperature in the presence of water-soluble carbodiimide. Unreacted groups in the sepharose were blocked by an additional reaction with acetic acid and the gel was washed extensively. The washed gel was packed into a column and tested for its ability to bind and purify the polyclonal antibody provided by Dr. Hunter.

Using this column, subsequent batches of ascietes fluid containing monoclonal anti T2 antibody provided by Dr. Hunter were purified, and titrated by EIA described earlier.

Production of Anti-idiotypic Antibody:

Three different immunization schedules were used to produce antidiotypic antisera in Balb/C mice:

1 Affinity purified monoclonal anti-T2 antibody coupled to KLH (Sigma).
2 Affinity purified monoclonal anti-T2 antibody coupled to affinity purified goat IgG (a gift from Dr. Finkelman).
3. Affinity purified monoclonal anti-T2 antibody coupled to monoclonal anti mouse IgD (B6 anti-BALB/C IgD).

The immunization schedule 2 was adopted following the successful use by Drs. Hunter and Finkelman in the production of original anti-T2 antibody. The hypothesis for this scheme is, when the conjugate is given along with purified goat anti-IgD, carrier specific helper - T Cells for goat IgG (which is conjugated monoclonal anti-T2 antibody) are stimulated. Since anti-T2 antibody will be a poor antigen being an isologous immunogen, it was reasoned that this immunization schedule can elect an anti-idiotypic response. The Schedule 3 was visualized with a hope that it will also recruit T-cell help for idiotypic determinants.

The three conjugates mentioned above were prepared using the method described by Bona et al. (1976). Briefly, equal parts of purified antibody and KLH or goat IgG, or B6 Anti-Balb IgD were mixed (0.5 mg/ml) in the presence of 0.05% glutaraldehyde. The reaction was carried until the mixture became opalescent, and then stopped by adding lysine to a final concentration of 0.05%. The resultant conjugate was extensively dialyzed. The immunization schedule followed, was as described by Ghenens et al. (1981). BALB/C mice (8-10 weeks) were injected with the conjugates emulsified in Freund's complete adjuvant. In the case of goat Ig coupled to the anti-T2, 50 mg of goat anti-IgD was given simultaneously. Each mouse was injected with 100 M of emulsion distributed among the hind foot pads, inguinal and axillary regions. Five days later a similar dose of antigenic preparation in Freund's incomplete adjuvant was given. The mice were boosted weekly with the respective conjugates in saline. The sera from these mice were examined for the presence of antidiotypic antibody, after 4th injection onwards.

The anti-idiotypic antibody was detected by EIA, using the monoclonal anti-T2 antibody coated 96 well plates and screening for a postive reaction and then for inhibition of that rection by free T2 toxin. Initially the serum samples were screened at a dilution of 1:100, using a fixed concentration of free T2 toxin such as 25 µg/ml for inhibition. The difference in OD units between the wells unblocked with T2 and those blocked with free T2 toxin was taken as the OD value demonstrating the anti-idiotypic antibody. The postive Id reaction was further confirmed using 96- wells plates coated with monoclonal antibody to paroxin (which is also IgG1), a gift by Dr. Hunter. Anti-idiotypic antibody was detected as early as after 4 injections. The titer of the antibody levels rose in accordance with the subsequent booster injections. However, no further increase was observed after 7 injections.

Of the three immunization schedules followed, mice receiving Schedule 3 and 1 demonstrated comparatively higher levels of anti Id antibody as early as after 4 injections, than those receiving Schedule 2, as determined by EIA. Mice receiving anti-T2 antibody coupled to B_6 anti BALB IgD (Schedule 3) have demonstrated a titer of 1:10,000 to 1:20,000 after 5th and 6th injection, which did not increase thereafter. Mice immunized with Schedule 1 had a titer

mice and rats. Attempts to fuse spleenocytes from mice immunized with Schedules 1 and 3 are in progress.

Standardization of Leucopenia in Mice:

To examine the efficacy of the anti-idiotypic antibody as a prophylactic agent, in vivo assays need to be established. Initially a single I.P. injection of varying concentrations of T2 toxin was used in the mice and their peripheral white cell counts were monitored for up to 4 weeks. As the results obtained by this route of dosing were not consistent, subsequently the oral route was investigated. The mice were orally dosed with T2 toxin in the concentration of 1 and 2 µg per gm body weight, and their white cell counts were monitored for various time periods up to a week (Fig. 1). Increase in blood white cells were obseved in both the dose level up to 96 hours and decreased there after. There was an initial drop in cell count after 3 hours of administration in both the cases. Drop in cell count persisted until 6 hours in case of 2 µg/gm group, and increased gradually until 96 hours and decreased later. The effect was less dramatic at 1 µg/gm level.

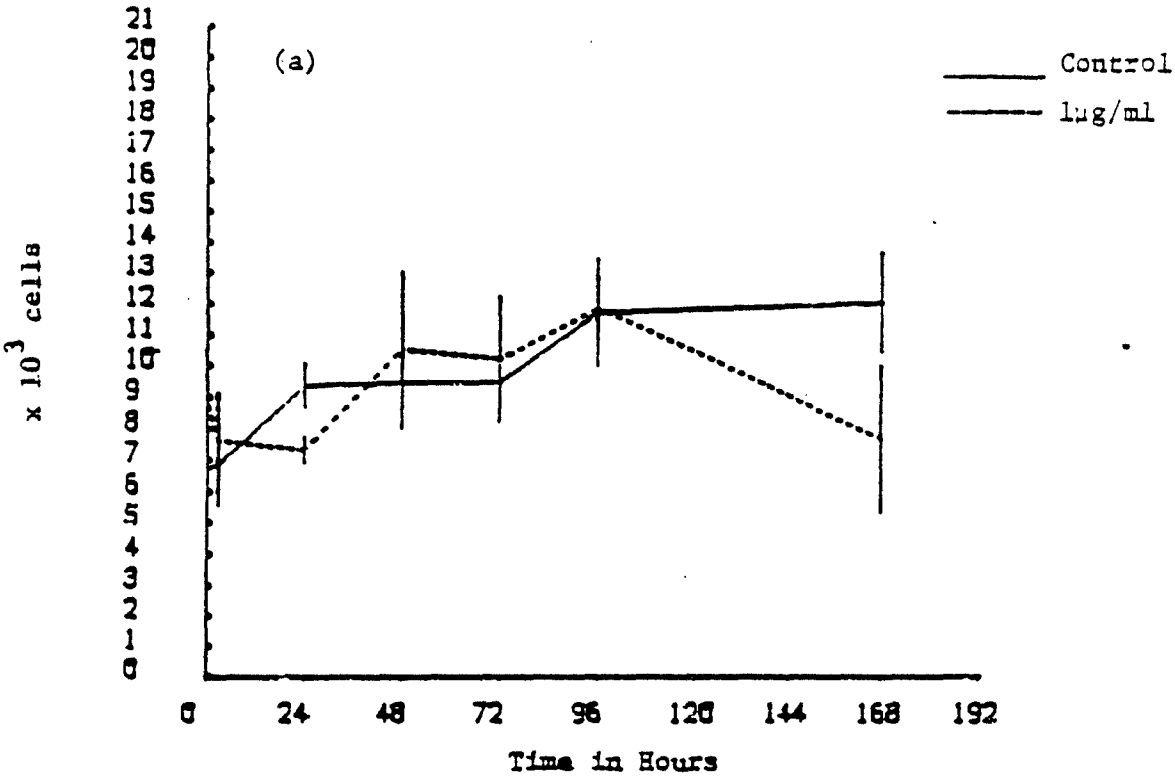
The rise in the WBC count in the peripheral blood is possibly due to the recruitment of lymphocytes from regional nodes spleen and other lymphatic organs. The mice orally dosed with 2 µg/gm, were also examined for, the total cell count in thymus and spleen at various time points (Fig. 2).There was a slight increase in cell population in the spleen at 1 hour after administration of the toxin, followed by a drop at 3 hours, which then increased until 24 hours, dropped again at 48 hours. At this point, WBC in the peripheral blood was high suggesting the migration of cells from lymphatics to the circulation. At 72 hours, there was a slight raise which decreased subsequently. A slightly different but similar pattern was observed in thymus cell counts. Initial drop at 1 hour was followed by an increase up to 6 hours, which decreased until 48 hours, slightly elevated at 72 and 96 hours, decreased thereafter.

Experiments are in progress to examine the morphology of the cell populations in these organs of the mice dosed with the toxin.

Future Directions

1. Expansion of immune mice producing anti Id antibody.
2. Fusion of spleenocytes from the above mice and production of hybridoma clones.
3. Extension of the immunization Schedules 1 to 3 into rats to produce xenogenic anti-Id antibodies and subsequently to use immune rat spleens to make xenogenic hybridoma.
4. Examination of sub populations of the cells in the lymphatic organs in mice dosed orally with T2 toxin.
5. Extension of leucopenia studies in rats.
6. Testing of therapeutic and prophylactic efficacy of monoclonal anti- Id antibody in mice and rats, against T2 induced leucopenia.
7. If the systemic treatment with monoclonal anti Id antibody is successful, the same regimen will be extended to the guinea pig skin necrotization model to test for protection against superficial effects.

Fig. 1. Induction of Leucopenia in BALB/C mice by T2-toxin (peripheral bloo



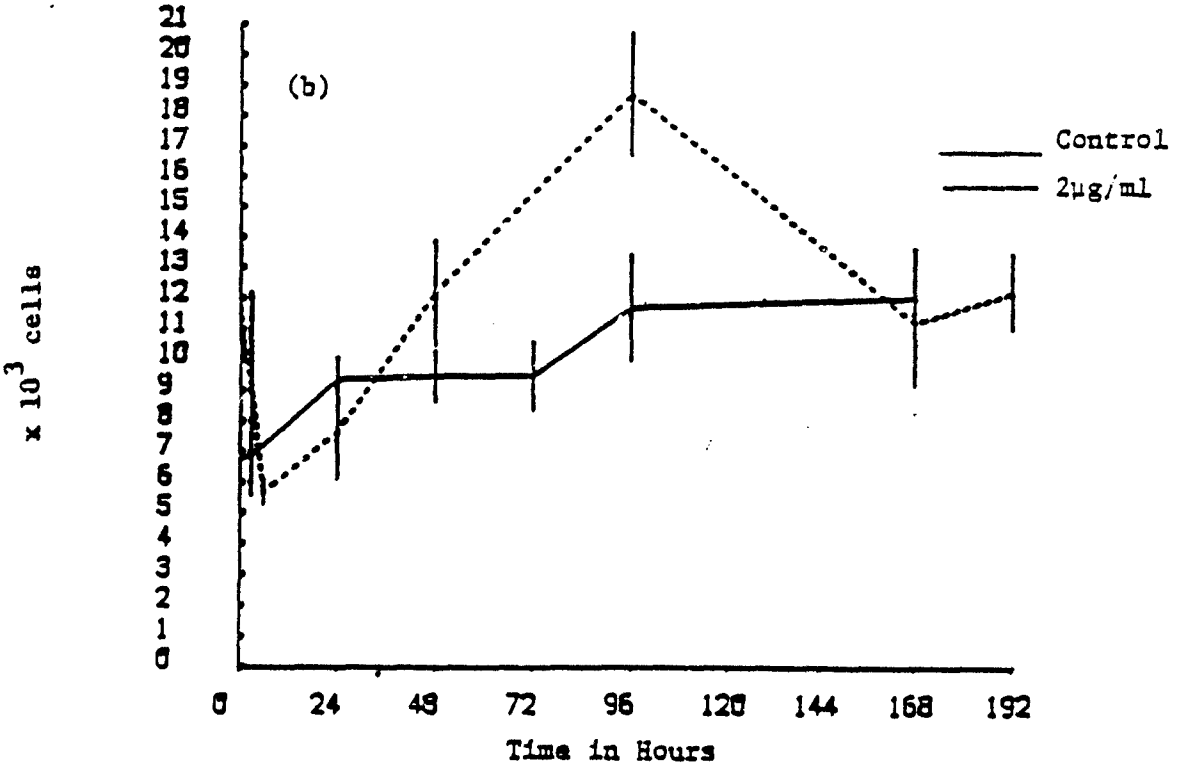
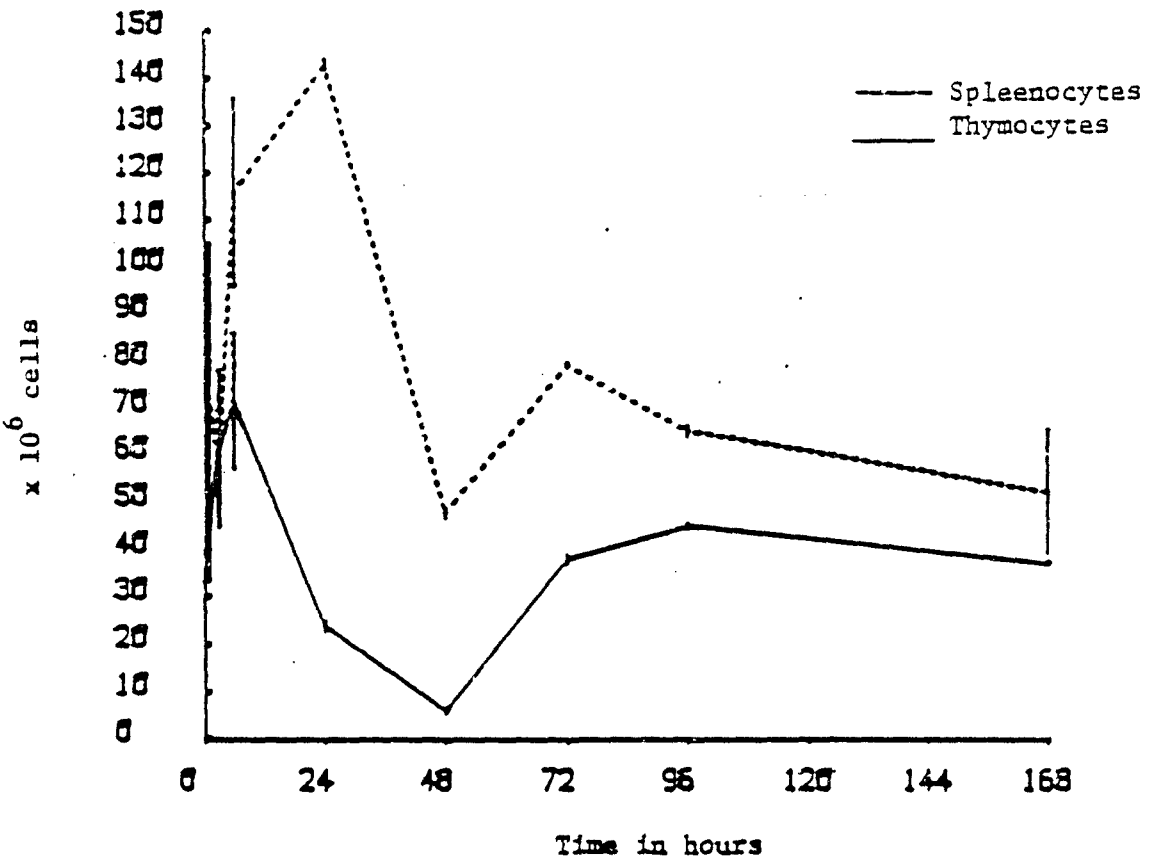


Fig. 2. Induction of Leucopenia in BALM/C mice
Effect of T2 toxin on thymus and spleen



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